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# REDUCTION OF SELENATE AND SELENITE TO ELEMENTAL SELENIUM BY A PSEUDOMONAS STUTZERI ISOLATE

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MICROMEDIA

# INTRODUCTION

Selenium is introduced into the environment by both industrial and agricultural activities. Agricultural drainage, smelter effluents, fly ash, sewage sludge and exhaust gases from the burning of fossil fuels are all sources of selenium (Doran, 1982; Larsen et al, 1989; McCready et al, 1990; Oremland et al, 1991). A number of selenium compounds are very toxic, however selenium is also an essential nutrient and low concentrations of selenium are required by many microorganisms (Doran, 1982; Stadtman, 1990).

Various microorganisms are capable of carrying out a number of transformations of inorganic selenium compounds such as reduction (Hudman and Glen, 1985; Maiers et al, 1988; McCready et al, 1966) oxidation (Sarathchandra and Watkinson, 1981) and methylation (Barkes and Fleming, 1974; Doran and Alexander, 1977). Although microorganisms capable of reducing the selenate ion have been isolated (Macy et al, 1989; Maiers et al, 1988; Oremland et al, 1989), selenite is more easily reduced than is selenate (Doran, 1982).

Recent work indicates that the dissimilatory reduction of selenium oxyanions may occur via a different pathway than that for sulfate reduction (Oremland et al, 1989; Steinberg and Oremland, 1990). Also in contrast to sulfate reduction, cell free extracts of Candida albicans have been shown to reduce the selenite ion to elemental selenium (Falcone and Nickerson, 1963; Nickerson and Falcone, 1963).

The objectives of this study were to characterize a

Pseudomonas stutzeri isolate capable of reducing the selenate ion to elemental selenium and to evaluate the effect of various parameters on the reduction rate.

## MATERIALS AND METHODS

### Bacterial strain and chemicals

The selenate and selenite reducing microorganism used in this study was provided by the U.S. Bureau of Mines in Salt Lake city. This strain was characterized by Gram stain and the oxi-ferm series of tests. It is a gram-negative rod that is oxidase negative. This strain was tentively identified in our Laboratory as Pseudomonas stutzeri and the identity was subsequently confirmed by Health and Welfare Canada, (Ottawa). The isolate was maintained on Trypticase Soy Agar (TSA)(Difco) to which sodium selenate was added. Sodium selenite and sodium selenate were obtained from Fluka. All other chemicals were of reagent grade.

### Preliminary experiments

Initial studies were carried out to evaluate the effects of shaking and inoculum size on the reduction of selenate. Inocula of 5 and 10% were used with and without shaking. An initial concentration of 200 ppm selenium was used.

### Optimum concentration of selenate and selenite

Prior to each experiment the bacteria were transferred from solid medium to Tryticase Soy Broth (TSB) for 24 h then a 5 % inoculum was transferred to TSB in order to obtain a standard

inoculum to begin each experiment.

The effect of concentration on reduction was evaluated for both selenium oxyanions. Varying amounts of filter-sterilized (0.2$\mu$m) sodium selenate and sodium selenite were added to 250 ml Erlenmeyer flasks containing 100 ml of TSB medium to obtain the following concentrations of selenium: 50; 100; 200; 300; 400; 500 and 600 ppm. Control flasks contained TSB only. All of the flasks were inoculated with the P. stutzeri isolate. At various time intervals ( 0; 24; 48 h) samples were taken for the determination of optical density (600 nm) and soluble selenium content. For the selenium analysis, 10 ml of the culture supernatant was filter sterilized (Nalgene units, 0.2 $\mu$m), acidified with HCl (4N) to prevent subsequent growth and kept at 4°C until they were analyzed by atomic absorption spectrophotometry (A.A).

The cultures were incubated in triplicate (pH 7.0) on a rotary shaker (150 rpm) at 30 °C until the next time of sampling.

Optimum temperature and pH

The effects of temperature and pH were evaluated using the previously described procedure and a selenium concentration of 200 ppm. The following temperatures were evaluated: 10; 20; 25; 30; 35 and 40 °C at pH 7. In order to examine the effect of pH, 4N HCl was added to TSB to obtain pH values of 5 and 6, and 4N NaOH was used to obtain pH values of 8 and 9.

Effect of different anions on the reduction.

A 1 M solution of each of the following salts were prepared: $Na_2MoO_4$; $Na_2SO_3$; $NaNO_2$; $NaNO_3$; $Na_2WO_4.2H_2O$ and $K_2CrO_4$. The solutions

were filter sterilized and varying amounts of these solutions were added to 250 ml Erlenmeyer flasks containing 100 ml of TSB medium which resulted in final concentrations of $10^{-4}$ M, $10^{-3}$ M and $10^{-2}$ M. Two ml of filter-sterilized sodium selenate or selenite were added. Controls contained only the selenium oxyanions and other flasks only the anions. The experimental conditions were: 30°C, pH 7.0, 200 ppm selenium and the flasks were agitated at 150 rpm.

Determination of the growth curve of Pseudomonas stutzeri.

The growth curve was measured in triplicate using 2800 ml Erlenmeyer flasks containing 1 L of TSB. The growth was determined in the presence of selenite, selenate and in the absence of both selenium salts. A 5% inoculum was used, a temperature of 30°C, 200 ppm of selenium where applicable and the flasks were shaken at 150 RPM. Samples were taken at various times for the measurement of optical density and selenium analysis by A.A.

## RESULTS

Aeration was found to be necessary for selenite and selenate reduction (Table 1) and all subsequent experiments were carried out using vigorous aeration. The size of the inoculum did not seem to affect the selenate reduction (Table 1). Selenium reduction occurred concurrently with exponential growth for both selenium oxyanions (Figures 1 and 2). The reduction of selenite was complete within 20 h and the complete reduction of selenate required only 14 h. The optical density (OD 600nm) in the presence of selenium was

higher than in the inoculated control (Figures 1 and 2). The reduction rate increased with increasing selenite concentrations up to a selenium concentration of 420 ppm (Table 2). A continuous increase in the rate of selenate reduction was observed up to a selenium concentration of 500 ppm (Table 2). Selenite reduction was optimal over the pH range of 7 to 9 with no reduction occurring below pH 6.0 (Table 3). Selenate reduction was somewhat more sensitive to pH as the lower limit for selenate reduction was pH 7.0. No reduction of selenate occurred at a temperature of 10°C but some selenite reduction occurred at that temperature (Table 4). The reduction rate of both anions increased rapidly between 20 and 25°C with the optimum temperature range occurring between 25 and 30°C. At 40°C, selenate reduction was somewhat lower.

The sulfate ion had no effect on either selenite or selenate reduction by P. stutzeri (Tables 5 and 6). Sulfite at concentrations between $10^{-3}$ and $10^{-4}$ M inhibited both selenite reduction and growth. Sulfite only inhibited selenate reduction at $10^{-2}$ M concentration. The nitrate and nitrite ions had no effect on growth, and selenite and selenate reduction. Tungstate had no effect on selenite reduction but in the presence of selenate both selenium reduction and growth were inhibited. At a concentration of $10^{-3}$ M the chromate ion completely inhibited growth, and selenite and selenate reduction, and at a concentration of $10^{-4}$ M greatly inhibited selenate reduction and growth. The molybdate ion had little effect on selenite or selenate reduction.

## DISCUSSION

This P. stutzeri isolate is interesting in that it rapidly reduces the selenate ion. Only a few microorganisms are capable of reducing selenate and their rates of selenate reduction are generally quite low. In most studies of selenate reduction mixed cultures or sediment samples rather than pure cultures are used to obtain selenate reduction (Larsen et al, 1989; Oremland et al, 1991; Steinberg and Oremland, 1990). Maiers et al (1988) isolated a bacteria that reduced selenate to selenite but one week was required for the complete reduction of 100 ppm of selenium in solution. Our isolate was capable of reducing 500 ppm of selenate in 24 h. From the deep red appearance of the medium during growth of our isolate, we have assumed that elemental selenium is the final end product, although no analyses were performed to confirm that observation.

The lag period for selenate reduction is longer than that for selenite reduction but the rate is more rapid once reduction commences (Fig.1 and 2). The toxicity of the selenite ion may be responsible for both the lower rate during log phase growth as well as the levelling off of selenite reduction at higher selenium concentrations (Table 2). The increased optical density compared to the controls which was observed in the flasks containing the selenium oxyanions was due to the contribution of the elemental selenium granules produced during reduction. The reduction of

selenate into elemental selenium is at least a two-step reaction in which selenate is reduced to selenite then possibly to Se (II) and eventually to red amorphous granules of elemental selenium (Altringer et al. 1989). Complete reduction of both seleno-oxyanions was not observed in our study probably due to the reoxydation of elemental selenium or to the presence of a third product.

The pH range for selenium reduction also corresponds to the pH range for the growth of pseudomonads (Table 3). Substrate limitation may have been responsible for the levelling off of the reduction rate above 25°C. In 24 h at 25°C, 84 and 89% of the selenite and selenate respectively had been reduced by the P. stutzeri isolate.

We investigated the effect of various anions on the reduction of seleno-oxyanions. It was found with Clostridium pasteurianum that sulfate acted as a competitive inhibitor of selenate uptake (Bryant and Laishley, 1988) but Oremland et al (1989) concluded in their studies that Se and S have different reductive biogeochemical cycles which is also in agreement with our results. The anions molybdate and chromate which are known to inhibit the reduction of sulfur oxyanions, did not prevent the reduction of the selenate in our studies. In the studies of Oremland (1990), chromate, tungstate, molybdate, nitrite and nitrate inhibited selenate reduction. They postulated that denitrification and selenate reduction may proceed by similar pathways, with the former inhibiting the latter. The inhibition of growth and selenate

reduction by $10^{-4}$ M tungstate is unusual (Table 6). Since no effect of tungstate was observed in the presence of selenite there seems to be a specific interaction between tungstate and selenate to prevent growth of the organism. The nature of the pathway for selenium reduction in the P. stutzeri isolate is as yet unknown.


## ACKNOWLEDGEMENTS

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## REFERENCES


1. Altringer, P.B., Larsen, D.M. and Gardiner, K.R. 1989. Bench scale process development of selenium removal from wastewater using facultative bacteria. pp. 643-657. in Biohydrometallurgy. J. Salley, R.G.L. McCready and P.L. Wichlacz (eds.). Proceedings of the International Symposium, Jackson Hole, Wyoming, August 13-18, 1989.

2. Barkes, L. and Fleming, R.W. 1974. Production of dimethylselenide gas from inorganic selenium by eleven soil fungi. Bull. Environ. Contam. Toxicol. 12: 308-311.

3. Bryant, R.D. and Laishley, E.J. 1988. Evidence for two transporters of sulfur and selenium oxyanions in Clostridium pasteurianum. Can. J. Microbiol. 34: 700-703.

4. Doran, J.W. 1982. Microorganisms and the biological cycling of selenium. pp. 1-32 in "Advances in Microbial Ecology" K. L. Marshall (ed). Plenum Press, New York.

5. Doran, J.W. and Alexander, M. 1977. Microbial transformations of selenium. Appl. Environ. Microbiol. 33: 31-37.

6. Falcone, G. and Nickerson, W.J. 1963. Reduction of selenite by intact yeast cells and cell-free preparations. J. Bacteriol. 85: 754-762.

7. Hudman, J.F. and Glenn, A.R. 1985. Selenium uptake by Butyrivibrio fibrifsolvens and Bacteroides ruminicola. FEMS

Microbiol. Lett. 27: 215-220.

8. Larsen, D.M., Gardiner, K.R., and Altringer, P.B. 1989. Biologically assisted control of selenium in process waste waters. pp. 177-185. in "Biotechnology in Minerals and Metal Processing" Scheiner, Doyle and Kawatra (eds). Society of Mining Engineers, Inc., Littleton, CO.

9. Macy, J.M., Michel, T.A. and Kirsch, D.G. 1989. Selenate reduction by a Pseudomonas species: a new mode of anaerobic respiration. FEMS Microbiol. Lett. 61: 195-198.

10. Maiers, D.T., Wichlacz, P.L., Thompson, D.L. and Bruhn, D.F. 1988. Selenate reduction by bacteria from a selenium-rich environment. Appl. Environ. Microbiol. 54: 2591-2593.

11. McCready, R.G.L., Campbell, J.N. and Payne, J.I. 1966. Selenite rduction by Salmonella heidelberg. Can. J. Microbiol. 12: 703-714.

12. Nickerson, W.J. and Falcone, G. 1963. Enzymatic reduction of selenite. J. Bacteriol. 85: 763-771.

13. Oremland, R.S., Hollibaugh, J.T., Maest, A.S., Presser, T.S., Miller, L.G. and Culbertson, C.W. 1989. Selenate reduction to elemental selenium by anaerobic bacteria in sediments and culture: Biogeochemical significance of a novel, sulfate-independent respiration. Appl. Environ. Microbiol. 55: 2333-2343.

14. Oremland, R.S., Steinberg, N.A., Presser, T.S. and Miller, L.G. 1991. In situ bacterial selenate reduction in the agricultural drainage systems of western Nevada. Appl. Environ. Microbiol. 57: 615-617.

15. Sarathchandra, S.U. and Watkinson, J.H. 1981. Oxidation of elemental selenium to selenite by Bacillus megaterium. Science 211: 600-601.

16. Steinberg, N.A. and Oremland, R.S. 1990. Dissimilatory selenate reduction potentials in a diversity of sediment types. Appl. Environ. Microbiol. 56: 3550-3557.

17. Stadtman, T.C. 1990. Selenium biochemistry. Ann. Rev. Biochem. 59: 111-127.

Table 1. Effect of aeration and inoculum size on reduction of selenate.

| Inoculum size | Shaking | Rate, µg/ml/h |
|---|---|---|
| 5% | + | 7.47 ± 0.24 |
| 5% | - | 0.29 ± 0.11 |
| 10% | + | 6.99 ± 0.10 |
| 10% | - | 0.43 ± 0.05 |

Table 2. Effect of selenium concentration on selenium reduction by *P. stutzeri*.

| Selenium salt | Initial Se Conc. (ppm) | Reduction Rate (μg Se/ml/h) |
|---|---|---|
| $Na_2SeO_3$ | 50 | 1.76 ± 0.12 |
| $Na_2SeO_3$ | 110 | 4.23 ± 0.13 |
| $Na_2SeO_3$ | 210 | 8.29 ± 0.29 |
| $Na_2SeO_3$ | 320 | 11.82 ± 0.25 |
| $Na_2SeO_3$ | 420 | 14.18 ± 0.15 |
| $Na_2SeO_3$ | 550 | 13.03 ± 1.11 |
| $Na_2SeO_3$ | 750 | 15.78 ± 0.87 |
| $Na_2SeO_4$ | 50 | 1.60 ± 0.07 |
| $Na_2SeO_4$ | 100 | 3.75 ± 0.35 |
| $Na_2SeO_4$ | 180 | 7.14 ± 0.10 |
| $Na_2SeO_4$ | 280 | 11.17 ± 0.13 |
| $Na_2SeO_4$ | 360 | 14.53 ± 0.34 |
| $Na_2SeO_4$ | 410 | 16.78 ± 1.79 |
| $Na_2SeO_4$ | 500 | 19.95 ± 0.24 |

Table 3. Effect of pH on selenium reduction by P. stutzeri.

| Selenium salt | pH | Reduction Rate (μg Se/ml/h) |
|---|---|---|
| $Na_2SeO_3$ | 5 | 0 |
| $Na_2SeO_3$ | 6 | 5.36 ± 0.54 |
| $Na_2SeO_3$ | 7 | 7.42 ± 0.19 |
| $Na_2SeO_3$ | 8 | 7.35 ± 0.33 |
| $Na_2SeO_3$ | 9 | 7.75 ± 0.18 |
| $Na_2SeO_4$ | 5 | 0.20 ± 0.06 |
| $Na_2SeO_4$ | 6 | 0.48 ± 0.03 |
| $Na_2SeO_4$ | 7 | 8.00 ± 0.15 |
| $Na_2SeO_4$ | 8 | 7.75 ± 0.48 |
| $Na_2SeO_4$ | 9 | 7.71 ± 0.23 |

Table 4. Effect of temperature on selenium reduction by P. stutzeri.

| Selenium salt | Temperature (°C) | Reduction Rate (μg Se/ml/h) |
|---|---|---|
| $Na_2SeO_3$ | 10 | 0.33 ± 0.23 |
| $Na_2SeO_3$ | 20 | 1.11 ± 0.24 |
| $Na_2SeO_3$ | 25 | 6.99 ± 0.02 |
| $Na_2SeO_3$ | 30 | 7.36 ± 0.17 |
| $Na_2SeO_3$ | 35 | 6.93 ± 0.17 |
| $Na_2SeO_3$ | 40 | 6.92 ± 0.11 |
| $Na_2SeO_4$ | 10 | 0 |
| $Na_2SeO_4$ | 20 | 3.53 ± 0.05 |
| $Na_2SeO_4$ | 25 | 7.40 ± 0.2 |
| $Na_2SeO_4$ | 30 | 7.25 ± 0.18 |
| $Na_2SeO_4$ | 35 | 7.08 ± 0.21 |
| $Na_2SeO_4$ | 40 | 4.03 ± 0.85 |

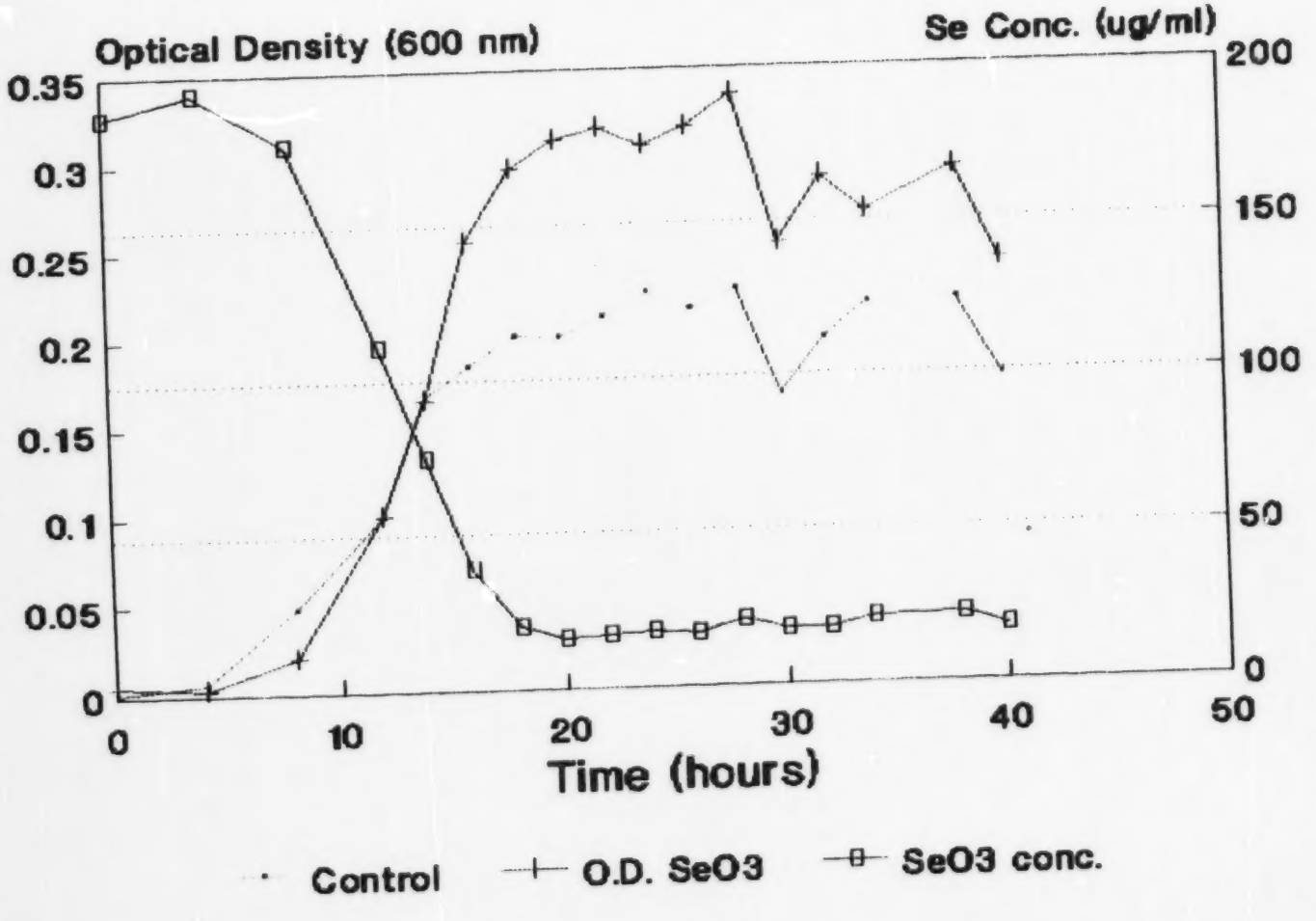
Table 5. Effect of different anions on selenite reduction by P. stutzeri.

| Anion | Concentration (M) | Reduction Rate (μg/ml/h) | Optical Density Time 24 hrs |
|---|---|---|---|
| None | None | 7.36 ± 0.17 | 0.248 ± 0.038 |
| $SO_4$ | $10^{-2}$ | 7.15 ± 0.16 | 0.317 ± 0.005 |
| $SO_4$ | $5x10^{-2}$ | 5.93 ± 0.27 | 0.256 ± 0.023 |
| $SO_3$ | $10^{-4}$ | 4.96 ± 0.85 | 0.253 ± 0.026 |
| $SO_3$ | $10^{-3}$ | 0.69 ± 0.17 | 0.123 ± 0.004 |
| $NO_3$ | $10^{-3}$ | 6.87 ± 0.48 | 0.312 ± 0.006 |
| $NO_3$ | $10^{-2}$ | 6.55 ± 0.86 | 0.269 ± 0.021 |
| $NO_2$ | $10^{-3}$ | 7.33 ± 0.44 | 0.292 ± 0.014 |
| $NO_2$ | $10^{-2}$ | 6.83 ± 0.40 | 0.293 ± 0.008 |
| $WO_4$ | $10^{-3}$ | 7.51 ± 0.43 | 0.343 ± 0.018 |
| $WO_4$ | $10^{-2}$ | 6.82 ± 0.17 | 0.313 ± 0.015 |
| $CrO_4$ | $10^{-4}$ | 7.13 ± 0.30 | 0.293 ± 0.003 |
| $CrO_4$ | $10^{-3}$ | 0 | 0.012 ± 0.005 |
| $MoO_4$ | $10^{-3}$ | 7.36 ± 0.56 | 0.303 ± 0.020 |
| $MoO_4$ | $10^{-2}$ | 6.63 ± 0.25 | 0.285 ± 0.017 |

Table 6. Effect of different anions on selenate reduction by P. stutzeri.

| Anions | Concentration (M) | Reduction Rate (μg/ml/h) | Optical Density Time 24 hrs |
|---|---|---|---|
| None | None | 7.25 ± 0.18 | 0.254 ± 0.021 |
| $SO_4$ | $10^{-2}$ | 7.01 ± 0.23 | 0.249 ± 0.010 |
| $SO_4$ | $5x10^{-2}$ | 6.56 ± 0.13 | 0.210 ± 0.020 |
| $SO_3$ | $10^{-3}$ | 6.53 ± 1.09 | 0.259 ± 0.005 |
| $SO_3$ | $10^{-2}$ | 0 | 0.019 ± 0.004 |
| $NO_3$ | $10^{-2}$ | 7.18 ± 0.36 | 0.280 ± 0.020 |
| $NO_2$ | $10^{-2}$ | 7.18 ± 0.19 | 0.292 ± 0.050 |
| $WO_4$ | $10^{-4}$ | 0 | 0.092 ± 0.020 |
| $CrO_4$ | $10^{-4}$ | 2.90 ± 1.59 | 0.163 ± 0.050 |
| $CrO_4$ | $10^{-3}$ | 0 | 0.012 ± 0.006 |
| $MoO_4$ | $10^{-3}$ | 7.08 ± 0.48 | 0.252 ± 0.020 |
| $MoO_4$ | $10^{-2}$ | 5.79 ± 0.27 | 0.201 ± 0.010 |

# Fig. 1 Selenite reduction by P. stutzeri



# Fig. 2 Selenate reduction by P. stutzeri